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A chicken GnRH analogue (Folligen) stimulates plasma testosterone level and sexual activity in the male marsh frog during and out of spawning season - Preliminary notes

by
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Abstract: The stimulatory effect of a new analogue of chicken I. gonadotropin-releasing hormone (Folligen) has been studied on specimens of marsh frog, *Rana ridibunda*. Folligen increased plasma testosterone (T) levels and induced breeding behaviour both during and out of the spawning season. Folligen (GnRHa) significantly enhanced plasma T levels in frogs tested in summer, and estradiol remained steady at high levels after the Folligen treatment. At the same time this hormone induced sexual activity of frogs in the postreproductive phase, too. According to our current and previous results, Folligen is suitable for stimulation of reproductive functions in lower classes of vertebrates.
Key words: *Rana ridibunda*, GnRHa, Folligen, sexual activity, testosterone

Introduction

Since the discovery of the porcine gonadotropin-releasing hormone structure four more molecular forms of GnRH have been isolated and characterised from vertebrate brains: chicken I, chicken II, salmon and lamprey forms (see King *et al.* 1989 for review). Two molecular forms of GnRHs have been identified in the brain of green frogs (*Rana esculenta* complex), these forms are closely related to salmon and chicken II. GnRHs (Cariello *et al.* 1989). Recently, the GnRH analogue (GnRHa), Buserelin [$_{D}$-Ser(But)^{6}desGly^{10}]-GnRH-ethylamide, has been used for studying the action of GnRH on the reproductive process (Pierantoni *et al.* 1984a, Fasano *et al.* 1988) in these species.

GnRH and its agonists can enhance the testicular function of frogs, either by stimulating the release of gonadotropins from the anterior pituitary (McCreery *et al.* 1982, Licht *et al.* 1984), or by directly enhancing the testicular androgen production (McCreery *et al.* 1982, Pierantoni *et al.* 1984a, Pierantoni *et al.* 1984b). Temperature and photoperiod also play an important role in the reproductive process, regulating androgen levels of male green frogs, *Rana esculenta*, through the central nervous system (Rastogi *et al.* 1978). Seasonal changes are well established in plasma steroid levels of green frogs in the natural environment (Polzonetti-Magni *et al.* 1984, Varriale *et al.* 1986). Testosterone (T) and dihydrotestosterone (DHT) showed annual fluctuation in the plasma. Testosterone increased significantly in January, and peaked in February and early March. DHT peaked in May-June, and a decrease could be found thereafter (Fasano *et al.* 1989). However, plasma estradiol peaked only in May (Varriale *et al.* 1986). Comparatively high estradiol levels were observed in male and female *Rana esculenta* specimens by Polzonetti-Magni *et al.* (1984) in May-July, and they suggested that

among several environmental cues this steroid may regulate post-reproductive refractoriness through the central nervous system by a negative feedback mechanism.

In recent years several new synthetic GnRHas have been developed in our laboratory and tested on frogs, fishes and mammals (Kéri *et al.* 1990, Gubányi *et al.* 1991). A chicken I. GnRH analogue [$_{D}$-Phe^{6},Gln^{8},desGly^{10}]-GnRH-ethylamide, named Folligen, was the most potent among them, which stimulated follicular maturation, ovulation and spermatogenesis. The scope of our investigation was to test the effects of Folligen (GnRHa) on testosterone and estradiol productions and sexual activity of *Rana ridibunda* in different seasons.

Material and methods

Animals and treatment: Adult male marsh frogs for summer and winter experiments (*Rana ridibunda* Pall., 65 gram±16 STD) were purchased from the Experimental Frog Farm (MITEX Kft, Lébénymiklós, Hungary) in 1989 and kept in small ponds before investigations. The frogs tested in spring were collected in the vicinity of Rétimajor during the spawning season in April, 1990. All the frogs used for experiments were fed by crickets (*Gryllus bimaculatus*) ad libitum. Female frogs were used for testing amplexing behaviour of male frogs in each experiment. Visually three different stages of sexual behaviour were investigated: chorusing of males, fighting between males and amplexing.

Winter experiment: Twenty male frogs, tested in winter were divided into two groups (10 individuals per group) and water temperatures gradually raised to 22±1 °C on 10 light/14 dark daylight regime. The first group was administered daily with GnRHa (Folligen) in the dorsal sac at 8 am for three days (1st day: 250 ng/g BW, 2nd day: 500 ng/g BW, 3rd day: 1000 ng/g BW). The second group was treated only with saline. Blood samples were taken on the third day at 2 pm.

Spring experiment: Ten male frogs, collected during the breeding season were treated daily (at 8 am) with GnRHa for three days (1st day: 0.25 ng/g BW, 2nd day: 0.5 ng/g BW, 3rd day: 1ng/g BW). The water temperature was between 21-22 °C. Blood samples were taken from every individual one hour before the first injection and 5 hours after the last treatment.

Summer experiment: 25 male frogs, tested in summer were grouped (10 specimens per treated groups and five individuals for controls). Blood sampling was carried out as in exp. 2. Groups were injected daily with 100 ng and 1 μg GnRHa per animal for six days, respectively. Controls were injected with saline. Frogs were kept at 19±1 °C and 10L/14D light-dark cycle.

Chemicals: The GnRHa, Folligen [$_{D}$-Phe^{6},Gln^{8},desGly^{10}]-GnRH-ethylamide was synthesized in our laboratory.

Blood sampling: Blood was collected from a blood vessel on a hind limb using a micropipette (Wijnands & van Gelder 1976). Samples were centrifuged (15 min, 4500 rpm). Thereafter, about 400-500 μl plasma was diluted with Ringer's solution up to one millilitre and stored at -25 °C until processing.

Hormone measurement: Determination of Plasma testosterone (T) and estradiol-17 (E) were carried out by radioimmunoassay (RIA) (Bodrogi & Fehér 1980, Poteczin *et al.* 1984).

Statistics: The significance of differences was evaluated using Student's T-test and Mann-Whitney U-test.

Results

Figure 1. summarizes the changes in plasma testosterone level (T) of males throughout the short-term studies (summer, winter and spring experiments). Plasma T levels were significantly raised by all GnRHa doses of all experiments ($P<0.05$ except 1 μg/animal/day treatment, $P=0.062$). No dose-dependent response of plasma T levels to different GnRH quantity was found in frogs tested in summer ($P=0.364$). The relative increment of plasma T was the greatest among frogs treated in winter (1620 %). GnRHa (Folligen) also increased plasma T levels in animals ready to mate in spring ($P<0.01$). Plasma estradiol levels (Fig. 2) did not show a significant decrease after the GnRHa administrations ($P>0.6$). Testosterone-estradiol ratios (Fig. 3) increased dramatically in GnRHa treated summer frogs ($P<0.05$).

Among frogs tested in summer, only those which were treated with 100 ng GnRHa/day showed sexual activity, such as calling and territorial behaviour (fighting). At the same time males were in amplexus with test females both in winter and spring experiments about 12 hours after the third injection.

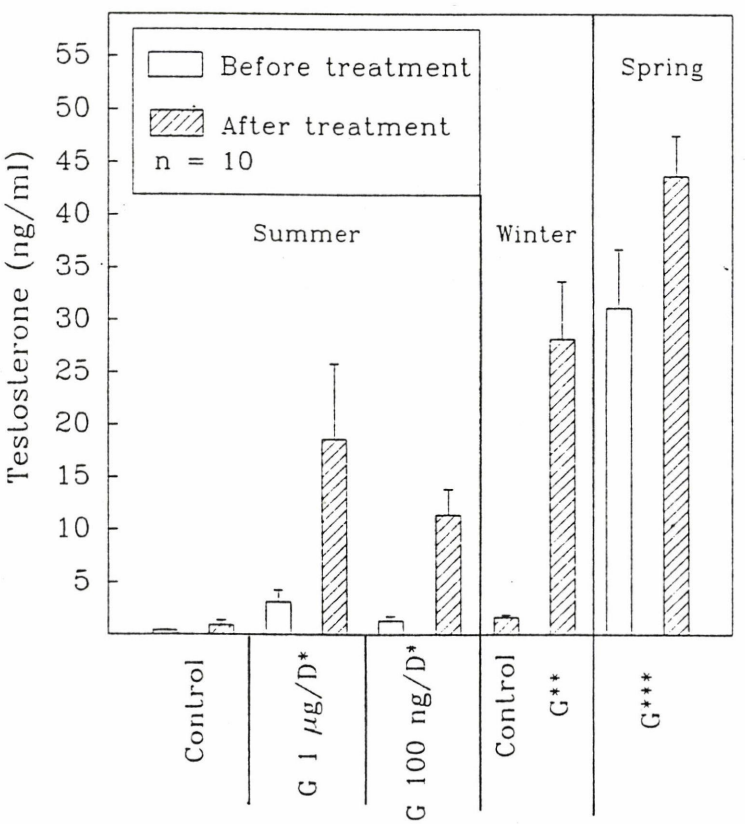

Fig. 1. Effect of different GnRHa treatment on plasma testosterone level in marsh frogs. Vertical bars represent means SEM (G = GnrHa; * = treatment lasted for 6 days; ** = frogs were treated with GnRHa for 3 days; 1st day: 250 ng/g BW, 2nd day: 500 ng/g BW, 3rd day: 1000ng/g BW, *** = frogs were treated with GnRHa for 3 days; 1st day: 0.25 ng/g BW, 2nd day: 0.5 ng/g BW, 3rd day: 1ng/g BW)

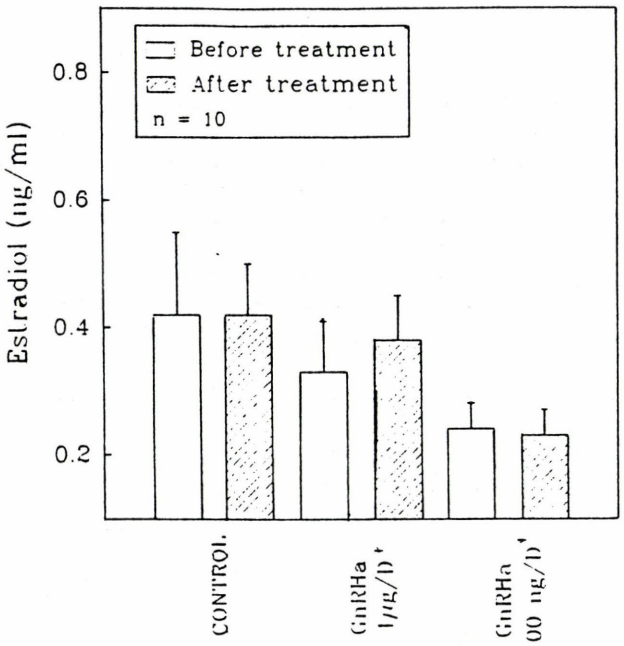

Fig. 2. Effect of different GnRHa treatment on plasma estradiol level in marsh frogs tested in summer.
Vertical bars represent means and SEM
(* = frogs were treated with GnRHa for 6 days, see Material and methods)

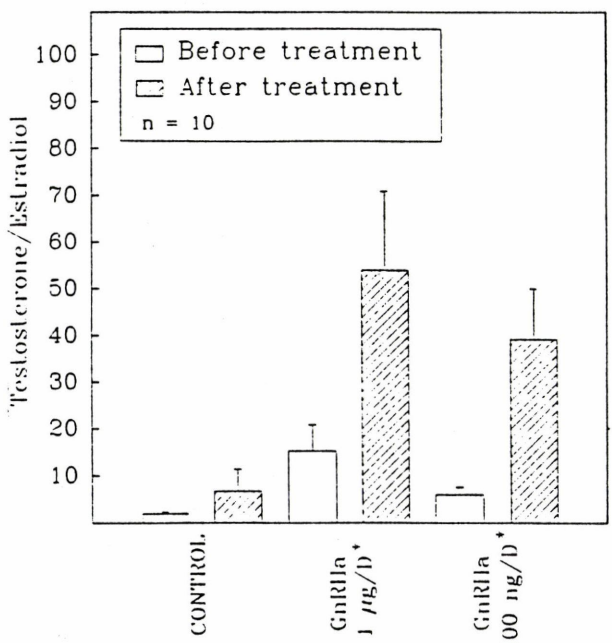

Fig. 3. Testosterone-estradiol ratios in marsh frogs tested in summer.
Vertical bars represent means and SEM
(* = frogs were treated with GnRHa for 6 days, see Material and methods)

Discussion

Our results demonstrate, that Folligen increased plasma testosterone concentration and induced sexual activity of marsh frogs in different seasons confirming and extending the experimental evidence about GnRH action on sexual steroid production in frogs (cf. McCreery *et al.* 1982, Pierantoni *et al* 1984a). To our surprise we found that Folligen (GnRHa) significantly enhanced plasma T levels in frogs tested in summer, without affecting the high plasma estradiol level. Estradiol remained steady at a high level after the Folligen treatment (cf. Polzonetti-Magni *et al.* 1984). It is difficult for us to interpret these findings since an interruption in the reproductive activity of "green frog complex" has been found during the summer (Polzonetti-Magni *et al.* 1984), resulting in a transitory refractoriness to environmental cues and causing unresponsiveness of frog testis to GnRH analogue (Buserelin) treatment and pituitary factors (Pierantoni *et al* 1984b). On the other hand McCreery & Licht (1984) suppose that testicular estradiol can inhibit the LH release, acting as a negative feedback. Taking these results into consideration, Folligen might affect testosterone production of the testis by modifying gonadotropin release of pituitary or by a possible direct gonadal action.

In this paper we have shown the stimulatory effect of a new GnRH analogue (Folligen) on plasma testosterone level and breeding behaviour of marsh frogs. The results of summer experiments with Folligen proved that this hormone is suitable for inducing sexual activity of frogs in the postreproductive phase, too.

In conclusion, also taking into account previous investigations with Folligen (Kéri *et al.* 1990, Gubányi *et al.* 1991), this chicken I GnRH analogue is very effective in stimulating reproductive functions in frogs and different classes of vertebrates.

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